Integrating next-generation dendritic cell vaccines into the current cancer immunotherapy landscape

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Abstract: Cancer immunotherapy is experiencing a renaissance spearheaded by immune-checkpoint inhibitors (ICIs). This has spurred interest in "upgrading" existing immunotherapies that previously experienced only sporadic success, such as dendritic cells (DCs)-vaccines. In this review, we discuss the major molecular, immunological and clinical determinants of existing first and second generation DC-vaccines. We also outline the future trends for next-generation DC-vaccines and describe their major hallmarks and pre-requisites necessary for high anticancer efficacy. Also, using existing data we compare DC-vaccines with ICIs targeting CTLA4, PD1, PD-L1, and argue that in various contexts next-generation DC-vaccines are ready to meet some challenges currently confronting ICIs; thereby raising the need to integrate DC-vaccines in future combinatorial immunotherapy regimens.

Dendritic cells as tools for anticancer vaccination

Dendritic cells (DCs) are the most efficient antigen-presenting cells (APCs) of the innate immune system [1, 2]. Conventionally, all APCs can present exogenously-captured antigens as major histocompatibility-complex (MHC) II-associated peptides (to $CD4^+$T cells) and endogenous antigens as MHC I-associated peptides (to $CD8^+$T cells) [3, 4]. However, DCs can accomplish both conventional presentation and cross-presentation (i.e. presenting exogenously captured antigens as MHC I-associated peptides) thereby eliciting more efficient $CD4^+$ and $CD8^+$T cell-activity [3, 4]. Productive activation of specific T cells not only requires a cognate antigen, called *signal 1*, but also co-stimulatory cues from ligands present on the APC surface e.g. CD80, CD86, CD40 (Figure 1) as well as pro-inflammatory cytokines, which constitute *signal 2 and 3*, respectively [5, 6]. The full-maturation of DCs is mandatory for the expression of molecules involved in s*ignals 2-3* [7]. *Signals 1-2-3* together ensure a type-1 immune polarization of $CD4^+$T cells (see Glossary) and efficient cytotoxic responses by $CD8^+$T cells (also termed, cytolytic T lymphocytes or CTLs) [7]. On the other hand s*ignal 1* delivered in absence of *signal 2*, and either absence of pro-inflammatory cytokines (*signal 3*), or presence of immunosuppressive cytokines, either elicits type 2 immunity (*via* Th2 cells; see Glossary) or immunosuppression (*via* Tregs; see Glossary) [3, 8].

Clinically relevant tumors actively disrupt these signals in the microenvironment (e.g. by directly inducing DC dysfunction, loss of tumor antigens, and eliciting higher production of immunosuppressive cytokines [9]) thereby limiting productive immune responses. [10]. These hurdles provided a rationale to produce vaccines based on patient-derived (autologous) DCs armed *ex vivo* with *signals 1-2-3* in order to facilitate *in situ* antitumor T cell immunity [7]. Of note, DC-vaccines might be better than other anticancer vaccination approaches like whole tumor cell-vaccines because they integrate several immunologically-relevant signals required for the efficient induction of antigen-directed T cell responses, on a single cellular platform. The basic principle behind DC-vaccines entails the isolation of autologous DCs from a patient followed by their *in vitro* 'loading' with appropriate source of tumor antigens (i.e. *signal 1*) and subsequent activation by defined 'maturation cocktails' (required to generate *signals 2-3*) [1, 11, 12]. These DC-vaccines are then injected back into the patient at an appropriate site in order to facilitate their homing towards the nearest lymph node for priming the T cells with the antigens they engulfed *in vitro*, in presence of proper co-stimulation [13, 14]. This priming facilitates T cell-driven anti-tumor immunity (full explanation of the DC-vaccines concept is provided in Figure 1). Various molecular and immunological determinants, on the levels of cancer cells, DCs or the patient/host/tumor (Figure 2, Key Figure), together regulate the efficacy of DC-vaccines e.g. the nature and source of tumor antigens, the composition of DC maturation cocktails, the impact of tumor burden or the tumor

immune microenviroment, the route/dose/scheduling of DC vaccination and the overall immunological fitness of the patient. These determinants are properly introduced and described in some details in Table 1. Recently, the immunological knowledge gained about DCs, tumor antigens, and tumor cell-DCs cross-talk has spawned new directions for creating "next-generation" DC-vaccines. In this review, we discuss the major features of the existing first and second generation DC-vaccines. We also outline the future trends for next-generation DC-vaccines and give particular attention to how these can be integrated into the current cancer immunotherapy landscape.

Dendritic cell-vaccines in the current cancer immunotherapy landscape

The field of cancer immunotherapy is currently experiencing a renaissance spearheaded by the success of immune-checkpoint inhibitors (ICIs; see Glossary) against selected solid tumors and Hodgkin's lymphoma, as well as by recent development in adoptive T cell-therapy (ACT; see Glossary) [15]. Besides establishing the clinical applicability of cancer immunotherapy, ICIs/ACTs have introduced revisions in how oncologists assess patient responses to immunotherapy [16, 17]. For instance, conventional clinical response criteria e.g. Response Evaluation Criteria in Solid Tumors (RECIST), have been reported to underestimate the therapeutic benefit of ICIs in ~15% of patients [16]. In fact, immune-related response criteria (irRC) demonstrated that positive patient responses are achieved by ICIs despite an initial RECIST-based negative outlook hallmarked by tumor 'pseudo-progression' [16]. However, it is also emerging that not all cancer patients respond to ICIs (applicable to at least ~50% patients [17, 18]) due to immunoresistance mechanisms including, but not limited to, extremely low tumor-infiltrating lymphocytes/TILs (applicable to 10-70% patients depending on cancer-type [18]), deregulation of immune-checkpoints, loss of tumor-antigens, monoclonality of response, mutations in interferon (IFN) signaling-axes or loss of sensitivity to IFN (see Box 1 for full details) [13, 17, 19-22]. Besides, severe-to-fatal side effects due to uncontrolled autoimmune responses remain a problem for ICIs/ACTs [23]. This has amplified the need to assess alternative or additional immunotherapy options that can be therapeutically beneficial for the subset of patients not responding to ICIs/ACTs.

These trends have spurred interest in "upgrading" existing immunotherapies that have previously failed to meet clinical expectations but still have the potential to confront various immuno-oncology challenges, and that may be used in combination with ICIs. On the top of this list is DC-vaccines. In the past, remarkable efforts have been invested in developing DC-vaccines with relatively modest clinical returns. Objective response rates (ORRs) to DC-vaccines in cancer patients have rarely exceeded ~15%; however it is worth noting that these estimates are based on the RECIST/WHO

criteria [12]. Ironically, most DC-vaccination clinical trials assessed RECIST/WHO-defined ORRs rather than overall survival (OS) or irRC-defined ORRs [12]. In assorted trials where OS was assessed, DC-vaccines could shift OS by ~20% - the threshold for clinically meaningful improvement [12]. Whether the lack of irRC-like assessment is behind the under-estimation of DC-vaccines' efficacy is unclear. Nevertheless, while the present generation of DC-vaccines is successful in increasing frequency/activity of TILs (in responding as well as few non-responding patients), they appear to fail to sustain their accumulation and effector function in non-responding patients [24, 25]. In the subsequent sections, we discuss the different features of past, present and future generations of anticancer DC-vaccines.

The past, present and future generations of DC-vaccines: experimental and clinical outlook

DC-vaccines have been applied against various malignancies (>200 clinical trials [26]), with the four most targeted cancer-types being, melanoma (>1000 patients), prostate cancer (>750 patients), glioblastoma (GBM; >500 patients) and renal cell carcinoma (RCC; >250 patients) [7, 12]. Figure 3a summarizes the most important historical landmarks in DC-vaccine development. Accordingly, DC-vaccines can be subdivided into three distinct "generations" (Figure 3b). First- and second-generation DC-vaccines will only be briefly discussed here. Instead, we will address in more details the emergence of next generation DC-vaccines and the outstanding future challenges of this field.

First- and Second-generation DC-vaccines

First-generation DC-vaccines (Figure 3b), consisted of either patient-isolated natural DCs or *ex vivo*-generated monocyte-derived DCs (moDCs) that were not matured further [12, 26, 27], a major reason behind their failure [26]. These DCs were loaded with either recombinant/synthetic antigenic-peptides (tumor-associated antigens/TAAs-derived, Table 1) or with tumor cell-lysates prepared through accidental necrosis [*via* freezing-and-thawing (F/T) or mechanical disruption] [26]. In spite of their limited success against RCC, melanoma and non-Hodgkin's lymphoma (tumor regression rate of only 3.3% in patients [26, 28, 29]), first-generation DC-vaccines established the safety and feasibility of DC-vaccines [12, 26, 27].

Second-generation DC-vaccines (Figure 3b) [7, 27], used mo-DCs that were fully matured *via* maturation cocktails (Figure 1, Table 1); and tumor antigens that consisted of recombinant/synthetic antigenic-peptides and tumor cell-lysates prepared *via* physical or mechanical disruption (e.g. necrosis) alone or followed by additional treatments (e.g. with irradiation like UV, X-rays or γ-rays and/or heat-shock) ensuring 100% cancer cell death i.e. avitalization and eliciting some

immunogenic (side-)effects [30]. Second-generation DC-vaccines have mostly (but not always) employed antigenic-peptides derived from tumor antigens like melanoma-differentiation antigens (e.g. MAGE antigens, MLANA, gp100), Wilms tumor 1 (WT1) and NY-ESO-1 [31, 32]. Besides this, other means of antigen-loading have also been implemented e.g. RNA/DNA-transfection and cancer cell-DCs (hybrid) fusions (Table 1) [7, 33]. Of note, evidence (across 173 clinical trials) indicates that 1711 patients treated with tumor cell-lysates based vaccines exhibit higher ORRs (8.1%) than 1733 patients that received antigenic-peptides based vaccines (3.6% ORR) - an observation providing some consensus on suitable tumor antigen source [18, 24, 34]. Second-generation DC-vaccines have performed better in the clinic than first-generation, by achieving ORRs in the 8-15% range, depending on the cancer-type [12]. Additionally, >50% cancer patients treated with second-generation DC-vaccines experienced antigen-specific CTL-activity or positive natural killer (NK)-cell responses [35, 36]. Also, second-generation DC-vaccines have increased median-OS by ~20% in many (but not all) studies [12].

Of note sipuleucel-T, also known as Provenge®, which is the only cellular vaccination product currently approved by US FDA for use in (prostate) cancer patients can be arbitrarily positioned at the intersection between the first and second generation DC-vaccines. This is because, it did not employ a defined DC 'maturation cocktail' (as is typical for second-generation DC-vaccines, Table 1) but rather relied on activation achieved by a fusion protein (PA2024) consisting of prostate antigen, prostatic acid phosphatase, fused to granulocyte-macrophage colony stimulating factor [37]. Moreover sipuleucel-T consisted of autologous peripheral-blood mononuclear cells (PBMCs) which included but were not limited to DCs. Sipuleucel-T has been able to prolong the OS of prostate cancer patients in some clinical trials, however it has largely failed to affect the time to disease progression [37].

Next-generation DC-vaccines

Clinical improvements from first-generation to second-generation DC-vaccines spurred interest in advancing towards next-generation DC-vaccines (Figure 3b). A major effort focuses on the nature and source of DCs. For instance, the use of specific subsets of naturally-occurring DCs is being employed since they confer two major advantages over mo-DCs i.e. better functionality and reduction in culturing time and costs [7]. Utilization of patient-derived DC-subsets has been made possible by some current technologies (e.g. antibody-coated magnetic beads) that allow rapid and enumerated isolation of natural DCs (>10 million DCs) [38, 39]. Of note, the superior functionality of this next-generation DC-vaccine approach originates from the use of more defined DC subsets specialized in MHC-I/II based antigen presentation and eliciting CTL responses (Figure 3c) [28]. For instance, plasmacytoid DCs (pDCs; see Glossary) are better at Type I IFN responses whereas

myeloid DCs (mDCs, see Glossary) are better in uptake of dead/dying cells and antigen presentation [40, 41]. Moreover, the use of BDCA1/CD1c^{+}mDCs (equivalent to murine cDC2) and BDCA3/CD141^{+}mDCs (equivalent to murine cDC1), two DC-subsets displaying high MHC-based antigen presentation capacity [11], would allow highly potent CTLs expansion and CD103^{+}CD8^{+} memory T cells' generation, respectively [26]. (Figure 3c).

Initial clinical trials show that next-generation DC-vaccines using naturally-occurring DC-subsets are not only safe and feasible but may also exhibit promising clinical efficacy [38, 39, 42]. For instance, a clinical study employing naturally-occurring, autologous, pDCs loaded with TAA-peptides, found that several melanoma patients administered with this vaccine experienced antigen-specific CD4^{+}/CD8^{+} T cell responses and exhibited a measurable IFN signature [38]. Similarly, another clinical study from the same investigators found that DC-vaccines based on naturally-occurring, autologous, CD1c^{+}mDCs induced long-term progression-free survival (12-35 months) in 4/14 melanoma patients [39]. One crucial point that is currently unclear from these clinical studies pertains to the superiority of a particular DC-subset over the others. This is decisive because, for instance, a recent study showed that murine cDC1 and cDC2 differentially activate CD8^{+}T cells and Th17 cells respectively [43]. On the level of vaccination, cDC1 and cDC2 based vaccines elicited anti-tumor CTLs and reduction in macrophages/myeloid cells-based immunosuppression within the tumor, respectively [43]. In a nutshell, different DC-subsets across human and mouse species differ in terms of their MHC-I/II based antigen presentation capabilities (Figure 3c). Thus it would be necessary, in near future, to initiate a multi-arm clinical trial in cancer patients that compares the efficacy of DC-vaccines based on different DC-subsets in order to irrevocably establish the DC-subset with highest capacity of eliciting TAA-specific T cell responses associated with prolonged patient survival.

It is clear that implementing the increased knowledge of DC biology has largely dominated and guided the transitions between different generations of DC-vaccines, whereas poor attention has been paid to the mechanisms of cancer cell death. On the other hand, in recent year the immunogenic potential of dead/dying cells has been emerging as a crucial definer of the anticancer vaccination-effect [44, 45]. Also, in last decades, the dogmatic view of necrosis being immunogenic and apoptosis being immunosuppressive has changed [44]. In fact accidental, i.e. genetically uncontrolled, necrosis can be poorly immunogenic [46]. In contrast, some instances of regulated cell death, being necroptosis or apoptosis, can be immunogenic [47, 48].

In particular, the molecular mechanisms underlying the induction of an immunogenic modality of apoptosis i.e. immunogenic cell death (ICD) (Figure 3d; see Glossary) [44, 45] have been characterized in considerable details, recently. Cancer cells undergoing ICD exhibit high

immunogenic potential owing to ordered exposure/release of a diversity of danger signals. These include- but are not limited to- surface-exposed 'eat me' signals that engage phagocytic receptors, 'find me' signals that facilitate recruitment of immune cells and other factors, which act as innate immune stimulators (see Glossary and Figure 3d for more details) (Figure 3d) [44, 45]. ICD-based anticancer vaccines are superior to F/T or mechanical necrosis-based vaccines [46, 49]. In fact, we recently demonstrated the high, danger signals-dependent, efficacy of ICD-based next-generation DC-vaccines against GBM [46]. ICD-based DC-vaccine synergized with the standard-of-care chemotherapy, to cause a Treg-to-Th1/Th17/CTLs shift in brain immune-microenvironment, leading to CTLs-driven anti-GBM immunity [46]. Interestingly, the ICD-derived Toll-like receptor (TLR)-agonist HMGB1, along with other danger signals, was crucial for DC-vaccine's efficacy despite presence of a TLR-agonist in the DC-maturation cocktail [46]. This highlights the vital role of endogenous danger signals for efficient DC-driven immunological responses.

Importantly, a clinical study showed that ICD-based DC vaccines exhibited clinical/immunological responses in 6/18 indolent B-cell lymphoma patients such that cancer cells of responders underwent better ICD (i.e. exposed/released higher DAMPs) than non-responders [50]. Of note, ICD is prone to contextual failures depending on factors like ICD-inducer types (Type I *vs.* Type II ICD; see Glossary), cancer-types or mechanisms compromising danger/immunogenic signaling (e.g. cancer cell-intrinsic low expression or defects in trafficking/exposure of danger signals, dysfunction of immunogenic phagocytosis, defects in danger signal-sensing on DC-level, such as loss-of-function polymorphism in *TLR4*) [14, 51]. Hence proper pre-selection of ICD-inducers tailored to specific cancer-types, and if possible the patient's genetic background, are crucial parameters for hitting the right "susceptibility zone". Moreover, chemical ICD-inducers (like anthracyclines) are not desirable for production of DC-vaccines compared to physical ICD-inducers (hypericin-photodynamic therapy/PDT, high hydrostatic pressure) because they either leave residual (active) drug concentrations behind or may exert cytotoxicity against DCs.

Apart from the above major strategies, the emerging relevance of neoantigens (i.e. tumor-specific antigens largely derived from nonsynonymous single nucleotide variations) in cancer immunotherapy has spurred interest in producing neoantigens-based next-generation DC-vaccines [52, 53]. In fact, a recent study found that neoantigen-loaded DCs generate potent MHC class I-restricted neoantigen-specific T cells (even *de novo*) in 3 melanoma patients; although survival advantage was not clear [54]. Interestingly some recent studies, utilizing antigen-based vaccination approach in absence of the autologous DCs, suggested that neoantigens-based vaccines may work in melanoma patients [55]. Nevertheless, it is not clear whether neoantigens are superior to TAAs/tumor cell-lysates. Potential drawbacks of this strategy include high frequency of

neoantigens being restricted to few cancer-types [7] and patient-to-patient neoantigen heterogeneity, making broad vaccine production difficult. Moreover, there is some concern that neoantigen-specific T cells generated against a mutated tumor protein via these approaches, may also cross-react with the non-mutated version of that protein thereby eliciting autoimmunity [56]. Nevertheless, more clinical insights are required to understand the potential efficacy of neoantigens based DC-vaccines.

Last but not least, although not encompassing DC-vaccines in *stricto sensu*, but rather the more general 'anticancer vaccines' category, it is worth noting that advances have also been made to activate DCs at the systemic level. Recently, intravenously administered RNA-lipoplexes encoding neoantigens or TAAs, have been shown to be efficiently captured *in situ* by DCs, thereby driving their maturation and consequent antigen-specific T cell responses, causing efficient tumor rejection [57].

Next-generation DC-vaccines: best combinatorial partner for immune-checkpoint inhibitors?

ICIs have autonomously achieved high clinical ORRs in cancer patients (Table S1 [58-74]), making it imperative to delineate the positioning of next-generation DC-vaccines within the future oncological paradigms.

Neoantigens-directed T cells are particularly active in tumors regressing following ICI-treatment [45, 53]. Neoantigen-generation is a highly stochastic process with low occurrence of immunologically-relevant neoantigens (so-called "neoantigen lottery") [75]. However, higher genomic mutational-burden results in higher neoantigen-burden which increases the probability of creating immunogenic neoantigens [53]. This caused the emergence of overall genomic mutational-burden as a (surrogate) predictive biomarker of positive ICI-responsiveness [53]. In fact, solid tumors with higher mutational load, such as melanoma or lung cancer, or overrepresentation of mutational lesions (e.g. microsatellite instability) have higher TILs [76] and are relatively superior responders to ICI treatment [17, 53, 75]. For example, if we compare the clinical ORRs achieved by ICI-treatment against various tumor-types (presented in Table S1 [58-74]) with respective tumoral mutational-burdens [77], it is indeed visible that cancer types with higher mutational-burden respond better to ICIs (Figure 4a) – a fact well-established in the literature [53, 76].

However neoantigen's burden is also possibly a limiting factor since, a subset-of-patients with low neoantigen's burden may not respond to ICIs [20, 75]. This creates an immunotherapy void that needs to be filled by finding an appropriate combinatorial counterpart for ICIs that can increase

TILs. Based on the available data trends, we propose that next-generation DC-vaccines can fill this void (Figure 4). For instance, recently a meta-analysis documented the average ORRs achieved by DC-vaccines across all published clinical trials related to melanoma (8.5%), prostate cancer (7.1%), GBM (15.6%) and RCC (11.5%) [12]. If we compare these ORRs achieved by DC-vaccines with respective tumoral mutational-burdens, a discordant scenario emerges (Figure 4a). Here, on one hand, the efficacy of DC-vaccines seems to be similar for two cancer-types with very different mutational-burdens i.e. melanoma and prostate cancer (Figure 4a). Yet, on the other hand, two cancer-types that respond most favorably to DC-vaccines i.e. GBM and RCC, also exhibit low tumoral mutational-burdens (Figure 4a). Overall this implies that, unlike ICIs, mutational burden may not be a reliable predictive biomarker for the efficacy of DC-vaccines.

Of note, to a certain extent GBM could be exceptionally susceptible to DC-vaccines as compared to some other cancer-types (Figure 4a). In fact, we have recently shown in preclinical settings, that while GBM is readily susceptible to next-generation DC-vaccines [46] yet it fails to strongly respond to ICIs targeting CTLA4, PD1 or IDO1 [77]. Moreover, most clinical predictive biomarkers of ICI-responsiveness forecast ICI resistant-phenotype in adult human GBM tumors (as compared to ICI-responsive cancer-types like melanoma and lung cancer) [77].

Moreover, if we compare the overall clinical-ORRs landscape for melanoma, GBM and RCC, an interesting picture emerges (Figure 4b). For decades, the ORRs achieved by chemotherapy (mainly dacarbazine) or radio-/chemo-therapy (mainly radiotherapy+temozolomide) regimens against melanoma and GBM, respectively, remained stagnant around or below 10% [12, 78]. For melanoma, DC-vaccines could not breach this 10%-ORR 'barrier' [12], which was eventually achieved by ICIs (Supplementary Table S1) [67]. However for GBM, the 10%-ORR 'barrier' has already been breached by the current, second-generation DC-vaccines (15.6%-ORR) (Figure 4b) [12]; a break-through initially achieved by anti-CTLA4 therapy for melanoma [67, 70]. Conversely the scenario is different for RCC. As opposed to melanoma and GBM, standard-of-care cytokine therapies alone (IFNα, IL2), targeted therapies alone (sunitinib, pazopanib) or combinations thereof (bevacizumab+IFN) have achieved promising ORRs (10-30%) against RCC [79]. DC-vaccines have performed fine against RCC (11.5% average-ORR) when compared to cytokine therapies (10-15% ORR) but relatively poorly when compared to targeted therapies (20-30% ORR) [12, 79]. On the other hand, while ICIs have achieved very promising ORRs in RCC patients, these have been relatively less compared to melanoma (Figure 4b) [80].

These analyses indicate the importance of DC-vaccines for at least some (but not all) tumors with low mutational-burden. This also makes biological sense, since tumors with low mutational-burden often have low TILs, a defect that DC-vaccines typically aim to correct [7, 46]. Overall the above

analyses also shows that each cancer type may exhibit unique patterns of susceptibility to different kinds of immunotherapies. Consequently, a single immunotherapeutic agent may not be globally applicable across all cancer patients. Henceforth, the application of next-generation DC-vaccines in combination with ICIs could be highly desirable for majority of patients of some cancer-types (e.g. GBM) or a subset-of-patients of other cancer-types (e.g. melanoma or RCC patients with low or negligible TILs).

Clinical reality for next-generation DC vaccines: Biomarker-driven combinatorial adjuvant treatment

The niche for next-generation DC-vaccines involves cancer patients prone to recurrence despite chemo-/radio-/targeted-therapy or ICIs/ACT. Such patients will have to be delineated after tumor de-bulking/regression in the primary-setting. In Figure 5 we outline a putative strategy for integrating DC-vaccines into future cancer immunotherapy approaches. We believe that the choice as well as the success of immunotherapy (including DC-vaccines) will be heavily driven by specific or broad predictive biomarkers that can reliably differentiate putative responders and non-responders [17]. The choice of these biomarkers would be in turn guided by the ever expanding knowledge on immune-resistance mechanisms [17] uncovered in respective non-responding patients (illustrative examples are indicated in Figure 5). Such predictive biomarkers could be detected on the level of peripheral blood (e.g. frequency of memory T cells, exosomes-specific markers) or tumor (e.g. Immunoscore, TILs-related metagene/protein signatures, immune-checkpoint status) [81, 82]. We elaborate the combinatorial cancer immunotherapy strategy integrating next-generation DC-vaccines below.

Once a clinically-diagnosable tumor has formed, it mainly represents a mass of highly immunoevasive cancer cells that have undergone immunoediting and successfully escaped cancer immunosurveillance (Figure 5). In this primary-setting, tumor regression, resetting of immunosuppressive micro-environment and/or induction of antitumor immunity is achievable through a first-line (standard-of-care) therapeutic regimen consisting of conventional therapies (chemo-/radio-/targeted-therapies), ICIs, ACT or a combination thereof (Figure 5). Choice of conventional therapies can be guided by their ability to induce immunogenic (side-)effect like abscopal effect (e.g. fractionated radiotherapy) and/or ICD (e.g. anthracyclines) [45]. Although setting demanding multiple cycles of (systemic) chemotherapy can be detrimental. For instance, chemotherapy-induced cancer cell death may fail to induce T cell-driven immunity due to severe lymphoablation caused by multiple cycles of chemotherapy [46, 83]. Properly executed

conventional treatments will either result in patients with no residual disease (creating long-term survivors/responders), or minimum residual disease (that doesn't yet represent clinical recurrence) with amenability towards immunotherapy. Simultaneously, various biomarkers like mutational-burden, immune-checkpoint expression patterns, antigen landscape of the tumor and degree of TILs (and their functional markers) can guide appropriate treatment combinations and/or sequence for primary immunotherapy. For example deciding, (i) between preference toward ICIs or ACT as first-line therapy (hence non-recipients for conventional therapy, Figure 5) or second-line therapy (i.e. non-responders to conventional therapy, Figure 5); or (ii) whether ICIs/ACT ought to be administered in concurrent combination with conventional therapies. Biomarkers can also help decide whether to skip ICIs or ACT entirely (non-recipients, Figure 5) owing to prediction of non-responsiveness or severe autoimmunity.

Patients that either fail to respond to above first-/second-line therapy options (non-responders, Figure 5) or are deemed unsuitable for ICIs/ACT (i.e. non-recipients, Figure 5), due to various resistance mechanisms including (but not limited to) low TILs, low immunogenic potential of cancer cells (in terms of danger signaling or MHC-defects) or low antigenicity (see Box 1) would be ideal for adjuvant application of next-generation DC-vaccines (Figure 5). However beyond these broad resistance mechanisms, there can also be specific resistance mechanisms that cannot be overcome by next-generation DC-vaccines alone. Such a scenario would demand combining DC-vaccines with other immunotherapies and/or targeted-therapies wherein the exact combination to be pursued would be guided by specific predictive biomarkers (Figure 5). Such biomarkers would be determined by known immune-resistance mechanisms (preclinically and clinically validated) (Figure 5). To this end, next-generation DC-vaccines can be combined with emerging ICIs (in case of tumors overexpressing assorted, context-dependent, immune-checkpoints), oncolytic viruses (e.g. owing to defects in Type I IFN signaling), ACT (in cases where ACT was not already given in primary setting; ACT can be especially vital for late-stage cancer patients that exhibit severe tumoral/systemic T cell-dysfunction [84]) and/or emerging targeted-therapies (Figure 5). Considering the fact that most antitumor T cells have low avidity T cell receptors (TCRs) against various TAAs [45], we believe that the dosages of DC-vaccines and other corresponding combinatorial therapies will have to be administered repeatedly in order to sustain long-term antitumor immunity (Figure 5).

Targeted-therapies to be combined with DC-vaccines can include (but may not be limited to), (i) therapies targeting oncogenic signaling-proteins e.g. $BRAF^{V600E}$-inhibitors, anti-Her2 antibodies, tyrosine-kinase inhibitors with some immunogenic properties like sunitinib/imatinib, and (ii) therapies depleting specific immune cell-types e.g. anti-CD25 antibodies against Tregs or

cyclooxygenase-2/arginase-inhibitors against myeloid-derived suppressor cells (MDSCs) [7, 27]. Interestingly, several clinical trials or studies are already exploiting some of the above discussed combinations (although not exactly with next-generation DC-vaccines) e.g. combination of chemotherapy+DC vaccines with cyclooxygenase-2 inhibitors or ACT has shown promise in melanoma/lung cancer clinical trials [7]. Similarly, combination of DC-vaccines with anti-CTLA4 ICI has been able to achieve 38% tumor response rate and 51% 6-month disease control rate [85]. Currently there are a number of clinical studies (according to the clinical trials database, www.clinicaltrials.gov) that are studying the combination of DC-vaccines with anti-PD1 ICIs or CAR-T cells/TILs-based ACT in cancer patients.

Concluding Remarks

Depending on the context (e.g. cancer-type, antigen heterogeneity/immunogenicity, tumor immune-microenvironment, general immunological fitness), DC-vaccines require various molecular or immunological hallmarks as pre-requisites to enforce efficient anti-cancer activity. The positive clinical response rates and safety profile of existing DC-vaccines set up a solid foundation for next-generation DC-vaccines to progress and address some of the challenges currently facing the immuno-oncology field. Next-generation DC-vaccines can be integrated in an adjuvant combinatorial treatment-setting at least for some cancer-types like GBM or RCC. While several challenges still need to be overcome (see Outstanding Questions), some lucid directions for creating next-generation DC-vaccines are available. Based on current concepts the most efficacious next-generation DC-vaccines could include loading specific naturally occurring DC-subsets with cancer cells undergoing ICD. Besides biological progress, technological innovations that ease regulation-compliant manufacturing hurdles are also necessary to increase affordability of DC-vaccines for patients, especially in combination with already costly ICIs and given the current strains on healthcare systems [17].

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Glossary:

Adoptive T cell-therapy (ACT): Consists of chimeric antigen receptor (CAR)-modified T cells/CAR-T cells, T cell receptor (TCR)-modified T cells or tumor-infiltrating lymphocytes/TILs. ACT involves deriving T cells either from peripheral source (CAR-T cells) or within tumor (TILs), expanding them *ex vivo*, activating them and/or genetically modifying them to increase recognition of particular (or multiple) tumor antigens; and then infusing them back into the patients.

Dendritic cell (DC)-subsets: In humans, two main DC-types exist i.e. myeloid-DCs (mDCs; also called classical/conventional-DCs) and plasmacytoid-DCs (pDCs; marked by BDCA2/4 and CD123). Here human mDCs are further subdivided into BDCA1/CD1c^{+}DCs, BDCA3/CD141^{+}DCs and CD16^{+}DCs. In mice, mDCs are subdivided as cDC1 (CD8α^{+}/CD103^{+}conventional-DCs; related to human BDCA3/CD141^{+}DCs), cDC2 (CD11b^{+}conventional-DCs; related to human BDCA1/CD1c^{+}DCs) (Figure 3c) [86]. Monocyte-derived DCs may also represent a distinct subset. Murine/human DC-subsets differ on several levels including MHC-I/MHC-II antigen presentation capacities and TLRs-diversity [11].

Immune-checkpoints inhibitors (ICIs): ICIs are mostly antibodies (but also small-molecules) that target immune-checkpoints to 'release the brakes' (like CTLA4/PD1/PD-L1) that form barrier to anti-tumor immunity [87]. Some emerging ICIs may target IDO1/TIM3/CD39/CD73/GITR/VISTA.

Immune-checkpoints: Immune-checkpoints are essentially 'immunological brakes' that tumor cells exploit to escape from antigen-directed T cells [45, 87]. Normally immune-checkpoints facilitate resolution of inflammation by exerting immunosuppressive effects and avoiding auto-immunity.

Immunogenic cell death (ICD): Cancer cells undergoing ICD liberate specific damage-associated molecular patterns (DAMPs) acting as danger signals and orchestrate immunostimulatory processes (like Type I IFN-response or CCL2/CXCL1/CXCL10 chemokines-based pathogen defense response-like 'altered-self' mimicry) (Figure 3d) [43-45]. DAMPs liberated by ICD include surface-calreticulin, surface-exposed/released heat shock proteins (HSPs), secreted-ATP, released-HMGB1 and released-nucelic acids [43-45]. These molecules together facilitate DC-based (immunogenic) phagocytosis of cell corpses and DC-maturation [51]. These fully-mature DCs, thereafter activate CD4^{+}T cells-driven CTLs/CD8^{+}T cells responses against residual disease (Figure 3d) [44, 45]. ICD-inducers are subdivided based on the nature of ER-stress driven danger signaling i.e. Type I ICD-inducers ('off-target' ER-stress e.g. anthracyclines, radiotherapy) and Type II ICD-inducers ('on-target' ER-stress e.g. hypericin-based photodynamic therapy/PDT) [45].

Objective response rates (ORRs): ORR is defined as the number/percentage of patients with a predefined quantitative reduction in tumor-size for a minimum time-period. Response duration is typically measured from the time of initial response until palpable tumor-progression.

Tregs: Regulatory T cells makeup ~10% of $CD4^+$T cells; they play a crucial role in resolution of inflammation and immune-homeostasis by negatively regulating autoreactive T cell-activity.

Type-1 immune polarization: This refers to a pro-inflammatory polarization characterized by phenotypic maturation/proliferation accompanied by production of immunostimulatory cytokines like IL12/IL6/IL1β/IFNα/IFNβ/IFNγ/TNF (e.g. Th1 cells, M1 macrophages).

Type-2 immune polarization: This refers to an anti-inflammatory polarization characterized by maintenance of immaturity or regulatory-ligands accompanied by production of immunosuppressive cytokines like IL10/TGFβ/PGE_2 (e.g. Th2 cells, M2 macrophages).

Box 1: Major molecular resistance mechanisms against immune-checkpoint therapy

De-regulation of tumor susceptibility to immune-checkpoints: This acquired resistance mechanism occurring in response to treatment with a particular ICI, entails most commonly the up-regulation of alternative immune-checkpoints, thus rendering the tumor resistant to that ICI; e.g. resistance to anti-PD1 therapy may occur in lung cancer due to up-regulation of an alternative immune-checkpoint, T-cell immunoglobulin mucin-3 (TIM-3) [19].

Loss of tumor antigens: A tumor micro-environment can select for cancer cells that have, through epigenetic or post-translational means, down-regulated the expression of major tumor antigens recognized by T cells thereby paving way for a 'cold' tumor phenotype on TILs-level.

Loss of sensitivity to interferons (IFNs): Chronic exposure of a tumor to IFNs (both Type I and Type II IFNs) can cause up-regulation of ligands for multiple T cell inhibitory receptors thereby giving rise to immune-resistant rather than immune-susceptible tumor phenotype [13].

Low tumor-infiltrating lymphocytes (TILs): Based on the density of TILs, tumors can be classified as "hot" (i.e. consisting of immunogenic immune-microenvironment marked by high TILs), "cold" (i.e. consisting of non-immunogenic immune-microenvironment marked by low TILs) or somewhere in between [20]. The density of TILs reflects the degree to which the immune system is recognizing and reacting against a tumor. Thus cancer patients with "cold" tumors/low TILs are likely to respond poorly to immunotherapy [20]. Beyond direct immunosuppression, TILs may also be excluded from a tumor due to stromal or vessel dysregulation.

Monoclonality of immune response: An effective anticancer immune response should be normally driven by various populations of T cell clones with affinity towards different types of cancer antigens [21]. Monoclonality or predominance of T cell clones directed towards one or few antigens can eventually limit the efficacy of immunotherapy if a tumor is successful in losing the expression of these antigens or restricting immune-access to them [17].

Mutations in IFN signaling axes: Genomic defects in IFN-γ pathway-associated genes have been found in patients not responding to ICIs, suggesting that tumors with loss of IFN-γ signaling are resistant to immunotherapy [22].

Figure Legends:

Figure 1. Principle behind dendritic cell (DC)-based cancer immunotherapy. Autologous $CD14^+$monocytes derived from peripheral blood of cancer patients (through leukapheresis) are differentiated with GM-CSF and IL-4/IL-13 into immature DCs *in vitro* [27]. Thereafter immature-DCs are supplied with tumor antigens, which can be derived from various sources [7] i.e. whole autologous/allogeneic cancer cell-lysates (induced to undergo cell death; require further antigen processing), recombinant antigenic-peptides (derived from tumor-associated antigens/TAAs or neoantigens; don't require antigen processing), RNA/DNA derived from cancer cells, recombinant RNA/DNA encoding defined-tumor antigens and DCs/tumor-fusion hybrids (provide stable antigen presentation). These antigen-loaded DCs are further matured/stimulated through "maturation cocktails" [27] consisting of specific cytokines (predominantly including TNF/IL1β/IL6/PGE_2; although PGE_2 represents a double-edged sword since it induces DCs' competent for lymph nodes-homing but also facilitates immunosuppressive Treg cells via IL12p70-suppression/IDO-stimulation) [27]. PGE_2-related problems may be overcome through toll-like receptor (TLR)-agonists although these can induce IDO [27]. These factors facilitate DCs-based MHC-I/MHC-II-driven antigen cross-presentation, presence of appropriate co-stimulation (CD80/CD86/CD83/CD40) and upregulation of lymph node-homing (via CCR7). Thereafter these fully-mature immunogenic-DCs are infused back into the patients where they migrate to the closest lymph node(s) and present the cancer-antigens to $CD4^+$/$CD8^+$T cells in presence of co-stimulation and T cell-stimulatory cytokines facilitating anti-tumor T cell polarization e.g. type-1/17 polarization (Th1/Th17) and cytotoxic T lymphocyte (CTL)-activity. CpG, 5'-C-phosphate-G-3'; F/T, freeze/thawing; FLT3L, FMS-like tyrosine kinase 3 ligand; GM-CSF, granulocyte monocyte-colony stimulating factor; IL, interleukin; LPS, lipopolysaccharides; MHC, major-

histocompatibility complex; PGE2, prostaglandin E2; Poly (I:C), polyinosinic:polycytidylic acid; TNF, tumor necrosis factor;

Figure 2. Schematic summarization of major molecular and immunological hallmarks for dendritic cells (DCs)-based anticancer immunotherapy. This plot depicts the 13 molecular and immunological hallmarks or pre-requisites that are most crucial for the efficacy of anticancer vaccination strategies (in various contexts) on the levels of cancer cells, dendritic cells (DCs) and the host or tumor (color coded). Utilization of some pre-requisites depends on context e.g. overall mutational load (surrogate for neoantigen load), loading of "pure" TAAs (i.e. recombinant peptides), blockade of immune checkpoints and usage of specific DC subsets may apply to some, but not all, cancer-types. Of note, cancer cell (*ex vivo/in vitro*) and host/tumor-level (*in vivo/in situ*) determinants are applicable to all anticancer vaccines in general whereas DCs-level determinants have dichotomous applicability i.e. *ex vivo* context for DC vaccines and *in situ* context for whole cancer cell-vaccines.

Figure 3. A timeline of major historical events in dendritic cell (DC)-vaccine's development, and their classification into different generations. (a) DC-vaccines' development commences from their discovery in 1973 when Zanvil Cohn and Ralph Steinman described them as rare murine splenocytes with distinctive tree-like morphology (dendritic derived from Greek word for tree i.e. dendreon) [27]. Several discoveries outlining the highly specialized role of DCs as antigen-presenting cells coupled with the discovery of human cancer antigens paved way for exploiting DCs for cancer immunotherapy [7, 88, 89]. The first clinical trial with DC-vaccines was published in 1996 followed by initiation of numerous clinical studies across many different cancer-types [89], including phase III clinical-trials in renal cell carcinoma/prostate cancer/glioblastoma/melanoma [27, 90]. These studies outlined the safety, efficacy and immunological or T cell responses associated with DC-vaccines [7, 90]. The field of DC-vaccines received a major boost in 2010, when sipuleucel-T (Provenge from Dendron Corporation) became the first cellular immunotherapy approved by US FDA [27]. Currently next-generation DC vaccines are in process of creation with some in early clinical phase. **(b, c, d)** Considering the historical development phases of DC-vaccines they can be convincingly divided into first-generation, second-generation and next-generation DC-vaccines **(b)** [currently being explored on the lines of different DCs subsets [11] **(c)** and immunogenic cell death or ICD **(d)** in cancer cells] based on developments on cancer cells/DCs-levels. CRT, calreticulin; HMGB1, high-mobility group box-1; HSP, heat shock proteins;

IFN, interferon; MHC, major histocompatibility complex; P2, purinergic-2; TLR, toll-like receptors;

Figure 4. The *status quo* for DC vaccines in the current, immune checkpoint inhibitor-dominated, cancer immunotherapy landscape. (a) A graphical representation of published objective response-rates (ORRs) clinically achieved by ICIs that have been averaged by pooling multiple studies for some cancer-types (see Supplementary Table S1) or average ORRs achieved by DC-vaccines (derived from a published meta-analysis [12]) against indicated cancer-types, *versus* published (representative) median somatic mutational-burdens of corresponding The Cancer Genome Atlas/TCGA tumor-types depicted below the ORRs graphs (derived from [77]). **(b)** ORRs-based (arbitrary) comparison of efficacy of conventional chemotherapy/radiotherapy, targeted therapy, cytokine therapy, DC-vaccines and specific ICIs against melanoma, glioblastoma and RCC. ACTs, adoptive T cell-therapy; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DC, dendritic cells; GBM, glioblastoma; HNSCC, head & neck squamous cell carcinoma; MHC, major histocompatibility complex; OV, ovarian cancer; PD1, programmed death-1; PDL1, programmed death-ligand 1; RCC, renal cell carcinoma; TCR, T cell receptors;

Figure 5. Intergating next-generation DC-vaccines into highly efficacious, biomarkers-driven, combinatorial regimens against cancer. Neoplastic cells usually undergo the process of cancer immunosurveillance consisting of cancer immunoediting i.e. initial immune-mediated elimination of neoplastic (recently transformed) clones (this is the point when the 'first' peak of anti-tumor immunity is reached), followed by their equilibrium with immune-resistant clones, ultimately resulting in escape of these latter clones, while the immune-susceptible ones are progressively eliminated accompanied by a 'global' suppression of anti-tumor immunity. Immune-resistant cancer cells that have escaped from immunosurveillance form the clinically-relevant tumors that are usually surgically resected upon clinical detection. Following surgery, the residual disease is usually treated with conventional therapies. Some patients may either receive conventional therapy in combinatorial regimens with ICIs/ACTs or receive ICIs/ACTs after failing to respond to former (i.e. non-responders). In cases where ICIs are first-line therapies, some patients do not receive conventional therapies first, hence they are labeled 'non-recipients'. Application of ICIs/ACTs can be either biomarker-driven or sometimes a standard-of-care. Patients that do not respond to these earlier regimens or receive conventional therapies but not ICIs/ACTs, and relapse (non-recipients in that context), can be routed for combinatorial regimens involving next-generation DC-vaccines

(i.e. adjuvant settings) and other highly efficacious immunotherapies or targeted-therapies whose rationale for combination can be biomarkers-driven. Of note, specific biomarkers may define presence of certain broad anti-tumor immunity resistance mechanisms. Following the ‘spikes’ in anti-tumor immunity achieved by ICD/ICIs/ACTs, these DC-vaccines based combinatorial regimens, if administered repeatedly can help maintain periodic increases in anti-tumor immunity thereby prolonging patient survival.

795 **Table 1. A systematic overview of the main molecular and immunological determinants of anticancer dendritic cell (DC)-vaccines.**

Determinants	Features	Relevance or strategy for integration	Current Challenges and Future Trends	Refs.
Tumor antigens	Loading of tumor antigens on immature DCs is the first step toward DC-vaccine production; Such antigens may include: - Tumor-specific antigens (encoded by mutated genes/neoantigens, viral genes or cancer-germline genes); - Tumor-associated antigens (encoded by tissue-specific genes or by genes that are overexpressed in tumor cells as compared to normal cells)	Antigens are loaded on DCs as: synthetic antigenic peptides, antigen-encoding RNA/DNA, autologous or allogeneic whole tumor cell-lysates,* tumor-derived whole RNA/DNA or tumor-DCs hybrids; - Antigenic peptides/defined RNA or DNA confer defined antigenicities (useful for cancer with known dominant antigens); - Whole tumor cell-lysates/RNA/DNA exploit the entire collection of tumor antigens (useful for cancer-types with antigenic heterogeneity/unknown antigens);	Heterogeneity of antigen expression in the tumor and the stimulation of T cells with low avidity are important roadblocks for DC-vaccines; Pulsing DCs with neoantigens (i.e. mutant antigens resulting from somatic mutations with less susceptibility to central/peripheral tolerance mechanisms), if they are known, may correct the latter problem;	[7, 24, 90-92] [24, 93]
DC maturation cocktail	DCs are activated through defined maturation-cocktails e.g. combination of pro-inflammatory cytokines (classically TNF+IL1β+IL6+PGE_2) and TLR-agonists; Various combinations of	More advanced strategies of DC maturation include (but are not limited to): - The α-type-1-DC-polarizing cocktail, TNF+IL1β+IFNγ+IFNα+poly(I:C); - TriMix-DCs electroporated with mRNA encoding constitutively-active TLR4, CD40L	- Certain cytokines/TLR-agonists (e.g. IL2, IFNα/γ, GM-CSF, bacterial-toxoids), co-stimulatory ligand-agonists (e.g. anti-4-1BB antibody) or oncolytic viruses can be co-administered with DC-	[7, 12, 26, 27, 33, 48, 94]

	cytokines/TLR-agonists are utilized by different DC-vaccine trials;	and CD70;	vaccines;	
Tumor burden and immune micro-environment	- Tumor-induced immunosuppression is a direct function of tumor burden.; - Immunosuppressive TILs or tumor microenvironment, predict poor survival of DC-vaccinated patients;	- Tumor-burden reduction *via* surgery and radio-/chemo-/targeted-therapies creates favorable conditions for DC-vaccines; - DC-vaccines exhibit higher clinical efficacy against minimum residual disease (i.e. adjuvant-settings) rather than primary tumor/metastasis (i.e. primary-setting);	- Loss/decrease in MHC-I expression or its components like β_2-microglobulin poses a major challenge; - Systemic depletion or type-1 immune polarization of TAMs, MDSCs and Tregs is desirable;	[1, 7, 10, 12, 17, 25, 27]
Route of DC-vaccination	Route of administration regulates DCs' access to lymph nodes.;	- Mostly administered intra-dermally near superficial lymph nodes; - Ultrasound-guided intra-nodal administration; - Intra-venous administration;	- Intra-dermal injection allows only ~5% DCs to reach the lymph nodes while intra-venous routes them to non-preferred locales e.g. lungs, liver, spleen, bone marrow; - Other routes like intranasal administration against mucosal tumors are also being tested;	[27, 95]
Dosage of DC-vaccines	DC dose positively associates with favorable prognosis;	Minimal effective DC dose per vaccine remains unclear** (0.3-3 million DCs per vaccine is apparently sufficient);	Further research is required to delineate the minimal effective DC dose per vaccine;	[7, 45, 54, 66, 87, 96-98]

Scheduling of DC-vaccines	Vaccines should be ideally administered in a repetitive manner to achieve maximal long-term efficacy;	Administration should be as early as possible following tumor regression and before relapse/recurrence (or in concurrence with minimal metastatic disease);	Tregs-driven immunosuppressive associated with multiple vaccinations is a potent challenge;	[7, 45, 54, 66, 87, 96-98]
Immunological fitness of patients	Immunological fitness is a classical determinant vaccine efficacy; Most markers for this are unclear but few are emerging e.g. gut microbiome and genetic-polymorphisms of immune-receptors (e.g. TLRs);	- Patients with immunodeficiencies (natural or induced by steroidal/lymphoablative-chemotherapy treatments) often fail to respond to DC-vaccination; - High incidence of MDSCs/Tregs predicts poor survival in DC-vaccinated patients;	Antibiotics, Cyclophosphamide or TLR-agonists (amongst others) can modulate overall immunological fitness;	[14, 24, 99]
*Autologous lysates are usually preferred over allogeneic lysates due to their higher immunological efficacy [18, 24, 92]. ** Interestingly a computational model proposed, ~85 antigen-presenting DCs to be sufficient for eliciting nodal T cell responses [96].				
Abbreviations: CD, cluster of differentiation; DC, dendritic cell; DNA, deoxyribonucleic acid; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; IL, interleukin; MDSC, myeloid derived suppressor cells; MHC, major histocompatibility complex; PGE, prostaglandin; RNA, ribonucleic acid; TAM, tumor associated macrophages; TIL, tumor infiltrating lymphocytes; TLR, toll-like receptor; TNF, tumor necrosis factor; Treg, regulatory T cell;				